IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of H. Van Urk, D.J. Mead, P.H. Morton, A.J. Cartwright, J. Cameron, D.J. Ballance, M.G.J. Grandgeorge, S. Berezenko, J.R. Woodrow, D. Sleep, and J-L.B. Veron

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(Atty. Docket No. P27,692 USA)

DECLARATION OF PHILIP MORTON UNDER 37 C.F.R. § 1.132

I, Philip Morton, hereby declare the following:

1. I am an inventor named on the above-identified application (hereafter "the present application").

2. I am presently Process Development Manager at Delta Biotechnology Limited, the assignee of the present application.

3. I have read the above-identified application and Applicant's Reply to the Office Action Dated November 14, 2005 including the experimental data provided within the Reply.

4. The experiments that provided the data were conducted under my supervision. I am familiar with the procedures used and the results obtained by the experiments.

5. The results demonstrate that partially purified albumin could be further purified by exposure to cation and anion exchange steps, wherein the cation exchange step is run in the negative mode with respect to albumin. In particular, it was found that the negative mode cation exchange step operated more efficiently (*i.e.* allowed the recovery of more

albumin and yet removed a greater proportion of impurities) when the albumin solution was highly concentrated. Moreover, the addition of octanoate to the albumin solution subjected to negative mode cation exchange chromatography results in the recovery of an increased yield of albumin, without affecting the level of contaminants. Additionally, increasing the operational pH, from 4.5 to 4.9, for an anion exchange step that is run in the negative mode with respect to albumin results in an increased loss of albumin. Experiments were conducted to confirm these results.

6. In the first experiment, different concentrations of an impure albumin solution were applied to a cation exchange chromatography column operated in the negative mode with respect to albumin. The albumin-containing cation exchange flow through was collected and the yield and contaminant levels were measured. The yield was determined by GP-HPLC. The detected contaminated level was measured by a Con A assay. The results of this experiment are shown in the following table.

Load Concentration	Albumin yield %	Detected Contaminant Level %
5 $g.L^{-1}$	38	0.17
25 $g.L^{-1}$	66	0.12
50 $g.L^{-1}$	63	0.07
100 $g.L^{-1}$	75	0.09

7. The results of the first experiment indicate that at higher load concentrations, negative mode cation exchange chromatography provides for more efficient recovery of albumin (as the load concentration increases from 5 g/L to 100 g/L the albumin recovery increases from 38% to 75%). Moreover, at the same time, higher load concentrations result in impurities being more efficiently removed (contaminant levels reduced from 0.17% to 0.09%).

8. In the second experiment, solutions of impure albumin were prepared with different concentrations of octanoate and applied to a cation exchange chromatography

column operated in the negative mode with respect to albumin. The albumin-containing cation exchange flow through and wash fractions were collected and the yield and contaminant levels were measured. The yield was determined by GP-HPLC. The detected contaminated level was measured by a Con A assay. The results of the experiment are shown in the following table.

Load octanoate concentration	**Albumin yield %**	**Detected contaminant level %**
4 moles.mole^{-1}	65	0.21
8 moles.mole^{-1}	73	0.23
12 moles.mole^{-1}	76	0.22

9. The results of this experiment indicate that including octanoate in the albumin solution that is applied to a cation exchange chromatography column operated in the negative mode with respect to albumin would have the advantageous effect of improving the albumin yield without compromising the purity of the product.

10. In the third experiment, solutions of impure albumin were prepared to the equivalent conductivity of 20mM or 40 mM Sodium acetate buffer at pH 4.5, 4.7 and 4.9. These solutions were applied to a DE-FF anion exchange chromatography column operated in the negative mode with respect to albumin and equilibrated in 20mM or 40 mM Sodium acetate buffer at pH 4.5, 4.7 or 4.9 depending on the load material. The albumin-containing anion exchange flow through was collected together with the wash and the yield was measured. The yield was determined by GP-HPLC. The results of the experiment are shown in the following table.

Buffer Type	**Operational pH**	**Recovery**
20mM Sodium acetate equilibration buffer	4.5	98%
20mM Sodium acetate equilibration buffer	4.7	70%
40mM Sodium acetate equilibration buffer	4.5	96%

40mM Sodium acetate equilibration buffer	4.7	91%
40mM Sodium acetate equilibration buffer	4.9	81%

11. The results of this experiment indicate that increasing pH from 4.5 to 4.9 results in a substantial reduction in albumin recovery from a negative mode anion exchange step (up to 30% loss at pH 4.7 using the 20mM Sodium acetate equilibration buffer) and it would be expected that a pH of 5.1 would lead to even greater losses of albumin than those observed in this experiment.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12-MAY-2006

Philip Morton

All about Albumin

Biochemistry, Genetics, and Medical Applications

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solution, seven to eight chlorides bind per albumin molecule (Fogh-Andersen *et al.*, 1993); NMR with ^{35}Cl indicated 10 or less (Halle and Lindman, 1978). Location and strength of binding are discussed later (Chapter 3, Section I,D,4, and Table 3-1). As the pH is lowered, chloride binding increases, to 11 ions/molecule at pH 5.2 and 22 at pH 4.2. Monovalent cations, sodium and potassium, are bound significantly only above pH 7.4.

The *isoelectric point*, in contrast to the *isoionic point*, is the pH at which the net charge of a molecule, including any bound ions, is zero. This is the pH at which a protein will not migrate in an electric field, as well as the pH zone in an isoelectric focusing gradient to which it will move and remain stationary. For undefatted albumin in 0.15 *M* NaCl the isoelectric pH is about 4.7 (Table 2-3); bound chloride and fatty acid ions cause it to be lower than the isoionic point.

At pH 7.4, the pH of blood, the net charge on the albumin molecule calculated from its amino acid composition is −15, −17, and −12 for HSA, BSA, and RSA, respectively (Table 2-1). This is also the relative order of anodal migration of these albumin species on electrophoresis at pH 7–9.

For HSA at pH 7.4, adding −7 for bound chloride ions, the net molecular charge becomes −22; with 42 g/L (0.64 m*M*) of albumin in plasma, the charge contributed by albumin is −14.1 mEq/L. (Bound fatty acid may raise this figure but bound calcium would lower it; see Chapter 3, Sections I,A and II,B). This is actually a little larger than the net charge of −12 on the total protein of plasma measured by Figge *et al.* (1991). These authors derived a formula for calculating the pH of plasma from the pO_2, the net strong ion (salt) charges, the inorganic phosphate concentration, and the albumin concentration. They concluded that albumin alone is significant as a net negative protein ion in plasma, accounting for the bulk of the clinically unmeasured anions. (The other normally unmeasured anions are carboxylates such as lactate and citrate.)

The titration curve of a protein is the composite curve of its many amino acid ionizable groups. The titration curve of albumin (Fig. 2-12d) shows several unusual features. For much of the information about the titration of albumins we are indebted to Tanford (1950), Steinhardt *et al.* (1971), and the review by Foster (1960), to which the reader interested in the development of equations from Debye–Hückel theory is referred.

The titration curve is flattest between pH 5 and pH 8, so that albumin is a rather weak buffer in the physiological pH range. Here it is mainly the imidazoles of the histidines and the terminal amino and carboxyl groups that are being protonated. The net charge is also affected slightly in this range by calcium binding. Figge *et al.* (1991) derived the HSA titration curve in the pH range 6.6 to 8.2 mathematically using the actual p*K* values for the 16 histidine imidazoles obtained from ^1H NMR (Bos *et al.*, 1989b), and showed that it closely agreed with the curve obtained by titration.

MOLECULAR BIOLOGY OF THE CELL

SECOND EDITION

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thus limit the approach of other macromolecules to the surface of the glycoprotein. In this way, for example, the presence of oligosaccharide tends to make a glycoprotein relatively resistant to protease digestion. It may be that the oligosaccharides originally provided an ancestral eucaryotic cell with a protective coat that, unlike the rigid bacterial cell wall, allowed the cell freedom to change shape and move. They may have since become modified to serve other purposes as well.

Proteoglycans Are Assembled in the Golgi Apparatus[50]

It is not only the *N*-linked oligosaccharide chains on proteins that are altered as the proteins pass through the Golgi cisternae en route from the ER to their final destinations; many proteins are also modified in other ways. As mentioned earlier, for example, some proteins have sugars added to selected serine or threonine side chains. This ***O*-linked glycosylation,** like the extension of *N*-linked oligosaccharide chains, is catalyzed by a series of glycosyl transferase enzymes that use the sugar nucleotides in the Golgi lumen to add one sugar residue at a time to a protein. Usually *N*-acetylgalactosamine is added first, followed by a variable number of additional sugar residues, ranging from just a few to 10 or more.

The most heavily glycosylated proteins of all are some *proteoglycan core proteins*, which are modified in the Golgi apparatus to produce *proteoglycans*. As discussed in Chapter 14 (see p. 806), this involves the polymerization of one or more glycosaminoglycan chains (long unbranched polymers composed of repeating disaccharide units) to serines on the core protein, with xylose rather than *N*-acetylgalactosamine added first. Many proteoglycans are secreted as components of the extracellular matrix, while others remain anchored to the plasma membrane as integral membrane proteoglycans (see p. 808). In addition, the mucus that is secreted to form a protective coating over many epithelia consists of a concentrated mixture of proteoglycans and heavily glycosylated glycoproteins (see, for example, Figure 8–60).

The sugars incorporated into glycosaminoglycans are heavily sulfated immediately after these polymers are made in the Golgi apparatus, which helps to give proteoglycans their high negative charge. The sulfate is added from the activated sulfate donor 3′-phosphoadenosine–5′-phosphosulfate (PAPS), which is transported from the cytosol to the lumen of a late Golgi compartment. A more subtle protein modification carried out in the Golgi is the addition of sulfate from PAPS to the hydroxyl group of selected tyrosine residues in proteins. Sulfated tyrosines are frequently found in secreted proteins and occasionally in the extracellular domains of plasma membrane proteins.

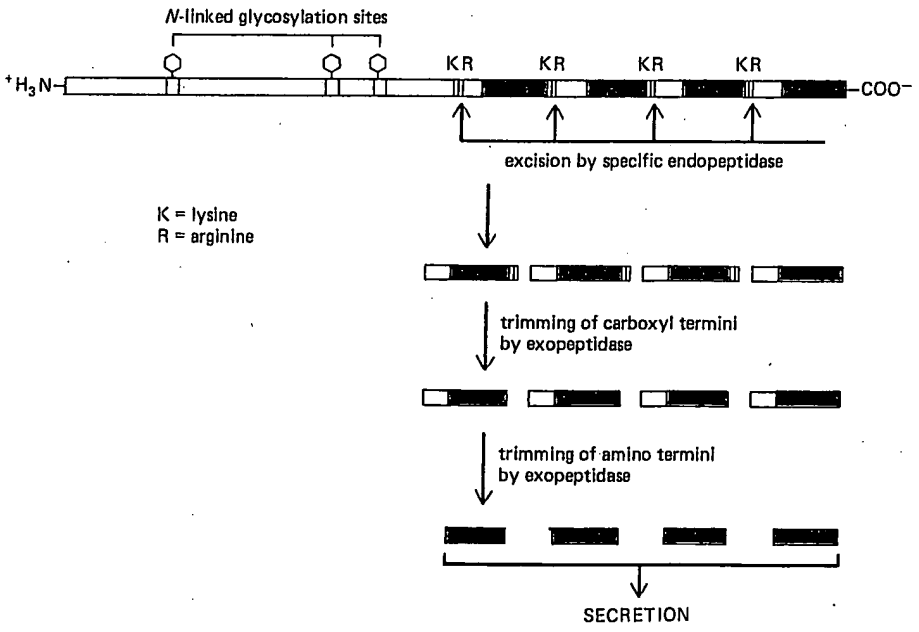

Figure 8–66 An example of a *polyprotein* that is cleaved to produce multiple copies of the same peptide signaling molecule. As indicated, the processing generally begins with cleavages at pairs of basic amino acids (here Lys-Arg pairs), which are catalyzed by a specific membrane-bound protease located in secretory vesicles or possibly in the *trans* Golgi network. Shown here is the processing pathway that produces the 13-amino acid α-factor in the yeast *Saccharomyces cerevisiae*, a secreted peptide that controls the mating behavior of this single-celled eucaryote. (After R. Fuller, A. Brake, and J. Thorner, in Microbiology 1986 [L. Lieve, ed.], pp. 273–278. Washington, D.C.: American Society for Microbiology, 1986.)

BIOCHEMISTRY

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To:

Our parents, who encouraged us,
Our teachers, who enabled us, and
Our children, who put up with us.

Cover Art: One of a series of color studies of horse heart cytochrome *c* designed to show the influence of amino acid side chains on the protein's three-dimensional folding pattern. We have selected this study to symbolize the discipline of biochemistry: Both are beautiful but still in process and hence have numerous "rough edges." Drawing by Irving Geis in collaboration with Richard E. Dickerson.

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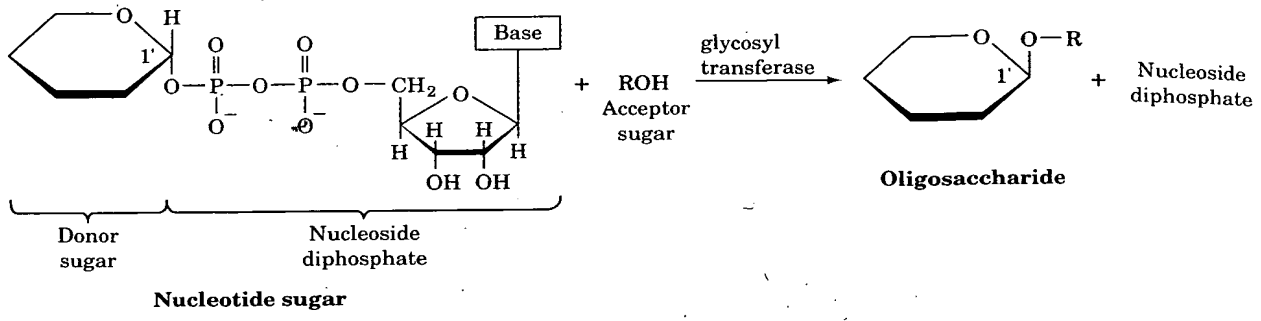

Figure 21-11
Nucleotide sugars are glycosyl donors in oligosaccharide biosynthesis catalyzed by glycosyl transferases.

Table 21-2
Sugar Nucleotides and Their Corresponding Monosaccharides in Glycosyl Transferase Reactions

UDP	GDP	CMP
N-Acetylgalactosamine	Fucose	Sialic acid
N-Acetylglucosamine	Mannose	
N-Acetylmuramic acid		
Galactose		
Glucose		
Glucuronic acid		
Xylose		

version of monosaccharide units to nucleotide sugars. A nucleotide at a sugar's anomeric carbon atom is a good leaving group and thereby facilitates formation of a glycosidic bond to a second sugar unit via reactions catalyzed by **glycosyl transferases** (Fig. 21-11). The nucleotides that participate in monosaccharide transfers are UDP, GDP, and CMP; a given sugar is associated with only one of these nucleotides (Table 21-2).

A. Lactose Synthesis

Several disaccharides are synthesized for future use as metabolic fuels. Typical of these is lactose [β-galactosyl-(1 → 4)-glucose; milk sugar], which is synthesized in the mammary gland by **lactose synthase** (Fig. 21-12). The donor sugar is UDP–galactose, which is formed by epimerization of UDP–glucose (Section 16-5B). The acceptor sugar is glucose.

Lactose synthase consists of two subunits:

1. **Galactosyl transferase,** the catalytic subunit, occurs in many tissues where it catalyzes the reaction of UDP–galactose and *N*-acetylglucosamine to yield *N*-acetyllactosamine, a constituent of many complex oligosaccharides (see, e.g., Fig. 21-16, Reaction 10).
2. **α-Lactalbumin,** a mammary gland protein with no catalytic activity, alters the specificity of galactosyl transferase such that it utilizes glucose as an acceptor, rather than *N*-acetylglucosamine, to form lactose instead of *N*-acetyllactosamine.

B. Glycoprotein Synthesis

Proteins destined for secretion, incorporation into membranes, or localization inside membranous organelles, contain carbohydrates and are therefore classified as glycoproteins. *Glycosylation and oligosaccharide processing play an indispensable role in the sorting and the distribution of these proteins to their proper cellular destinations.* Their polypeptide components are ribosomally synthesized and processed by addition and modification of oligosaccharides.

The oligosaccharide portions of glycoproteins, as we have seen in Section 10-3C, are classified into two groups:

1. ***N*-Linked oligosaccharides,** which are attached to

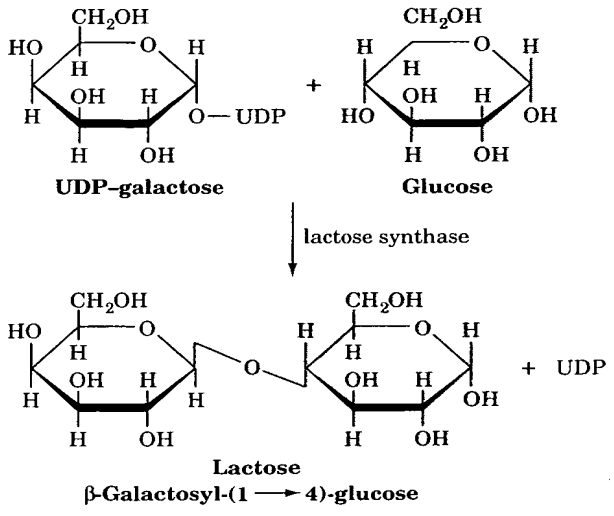

Figure 21-12
Lactose synthase catalyzes the formation of lactose from UDP–galactose and glucose.

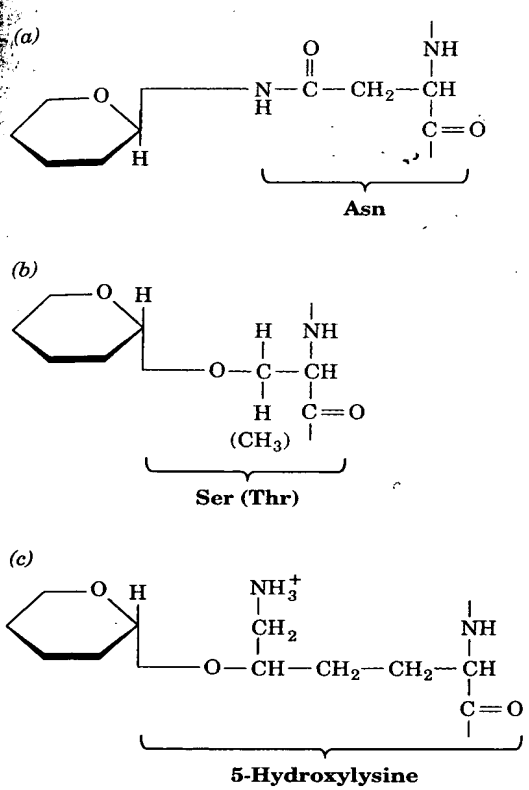

Figure 21-13
Types of glycosidic bonds in glycoproteins. (*a*) *N*-Linked glycosidic bond to an Asn residue in the sequence Asn-X-Ser/Thr. (*b*) *O*-Linked glycosidic bond to a Ser (or Thr) residue. (*c*) *O*-Linked glycosidic bond to a 5-hydroxylysine residue in collagen.

their polypeptide chain by a β-*N*-glycosidic bond to an Asn residue in the sequence Asn-X-Ser or Asn-X-Thr, where X is any amino acid residue except Pro or perhaps Asp (Fig. 21-13*a*).

2. ***O*-Linked oligosaccharides,** which are attached to their polypeptide chain through an α-*O*-glycosidic bond to Ser or Thr (Fig. 21-13*b*) or, only in collagens, to 5-hydroxylysine residues (Fig. 21-13*c*).

We shall consider the synthesis of these two types of oligosaccharides separately.

N-Linked Glycoproteins Are Synthesized in Four Stages

N-Linked glycoproteins are formed in the endoplasmic reticulum and further processed in the Golgi apparatus. Synthesis of their carbohydrate moieties occurs in four stages:

1. Synthesis of a lipid-linked oligosaccharide precursor.
2. Transfer of this precursor to the NH_2 group of an Asn residue on a growing polypeptide.
3. Removal of some of the precursor's sugar units.
4. Addition of sugar residues to the remaining core oligosaccharide.

We shall discuss these stages in order.

N-Linked Oligosaccharides Are Constructed on Dolichol Carriers

N-Linked oligosaccharides are initially synthesized as lipid-linked precursors. The lipid component in this process is **dolichol,** a long-chain polyisoprenol of 14 to 24 isoprene units (17–21 units in animals and 14–24 units in fungi and plants; isoprene units are C_5 units with the carbon skeleton of isoprene; Section 23-6A), which is linked to the oligosaccharide precursor via a pyrophosphate bridge (Fig. 21-14). Dolichol apparently anchors the growing oligosaccharide to the endoplasmic reticulum membrane. Involvement of lipid-linked oligosaccharides in *N*-linked glycoprotein synthesis was first demonstrated in 1972 by Armando Parodi and Luis Leloir who showed that, when a lipid-linked oligosaccharide containing [^{14}C]glucose is incubated with rat liver microsomes (vesicular fragments of isolated endoplasmic reticulum), the radioactivity becomes associated with protein.

N-Linked Glycoproteins Have a Common Oligosaccharide Core

The pathway of dolichol-PP-oligosaccharide synthesis involves stepwise addition of monosaccharide units to the growing glycolipid by specific glycosyl transferases to form a common "core" structure. Each monosaccharide unit is

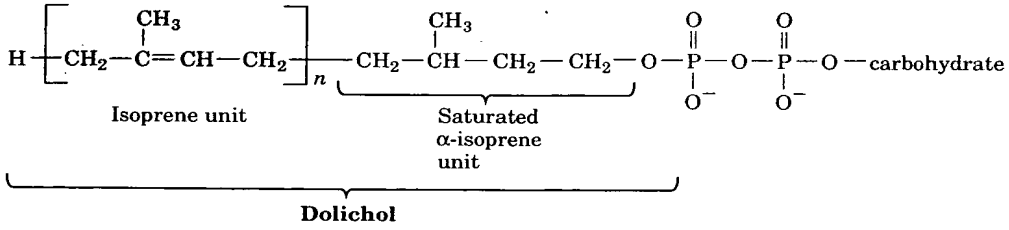

Figure 21-14
The carbohydrate precursors of *N*-linked glycosides are synthesized as dolichol pyrophosphate glycosides. Dolichols are long-chain polyisoprenols (n = 14–24) in which the α-isoprene unit is saturated.